ORIGINAL ARTICLE

Mutation Profiling Impacts Clinical Decision Making and Outcomes of Patients with Solid Pancreatic Lesions Indeterminate by Cytology

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ABSTRACT

Introduction Indeterminate cytology occurs in a significant number of patients with solid pancreaticobiliary lesion that undergo endoscopic ultrasonography fine needle aspiration or endoscopic retrograde cholangio-pancreatography and can incur further expensive testing and inappropriate surgical intervention. Mutation profiling improves diagnostic accuracy and yield but the impact on clinical management is uncertain. We determined the performance of mutation profiling in clinical practice and its impact on management in solid pancreaticobiliary patients with indeterminate cytology. **Methods** Solid pancreaticobiliary patients with non-diagnostic, benign, atypical or suspicious cytology who had past mutation profiling testing were included. Mutation profiling examined KRAS mutation and a tumor suppressor gene associated loss of heterozygosity mutation panel covering 10 genomic loci. Two endosonographers made management recommendations without and then with mutation profiling results, indicating their level of confidence. **Results** Mutation profiling improved diagnostic accuracy in 232 patients with indeterminate cytology. Among patients with non-diagnostic cytology, low risk mutation profiling provided high specificity and negative predictive value for the absence of malignancy while high risk mutation profiling identified malignancies otherwise undetected. Mutation profiling increased clinician confidence in management recommendations and resulted in more conservative management in 10% of patients. Mutation profiling increased the rate of benign disease in patients recommended for conservative management (84% to 92%, $p<0.05$) and the rate of malignant disease in patients recommended for aggressive treatment (53% to 71%, $p<0.05$). **Discussion** Mutation profiling improved diagnostic accuracy and significantly impacted management decisions. Low risk mutation profiling results increased recommendations for conservative management and increased the rate of benign outcomes those patients, helping to avoid unnecessary aggressive interventions and improve patient outcomes.

INTRODUCTION

The current standard diagnostic modality for solid pancreaticobiliary lesions (SPL) is an endoscopic ultrasound (EUS) guided fine needle aspiration for cytological evaluation. A recent meta-analysis has shown that although cytology has a high sensitivity (85%) and specificity (98%) for detecting malignancy in SPLs, the negative predictive value (NPV) is only 65% [1]. It has also been reported that despite improvement in needle devices such as introduction of EUS needles capable of taking core samples, and also, widespread use of on-site rapid cytology evaluation, in a significant number of patients the cytology samples are reported indeterminate or non-diagnostic due to limited cellularity [2]. This typically results in further expensive testing, such as repeat sampling, delayed diagnosis, missed diagnosis and even inappropriate surgical intervention for confirmation of diagnosis.

Advances in molecular diagnostics have led to an increase in available testing options for diagnosing malignancy in SPLs. Multiple studies have shown that mutation profiling (MP) for markers such as KRAS, MUC, p53, p16, S100P, SMAD4 and profiling of microRNAs can improve diagnostic sensitivity and specificity, especially in borderline cytological cases of suspected malignancy [3, 4, 5, 6, 7, 8]. These studies examined molecular changes in normally discarded, cell-free supernatant fluid and/or micro-dissected cytology slides obtained by standard EUS FNA and ERCP procedures. However, most of these studies were exploratory and limited by small sample size, and importantly, none of these studies demonstrated an impact of molecular results on clinical decision making. Though initial results are promising, further studies are needed to demonstrate the diagnostic capabilities of MP on standard specimens of SPLs in a larger cohort of patients in non-exploratory clinical practice.

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Abbreviations ERCP endoscopic retrograde cholangio-pancreatography; EUS endoscopic ultrasonography; FNA fine needle aspiration; MP mutation profiling; SPL solid pancreaticobiliary lesion
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Our study examined the ability of MP to aid in the diagnosis of adenocarcinoma in SPLs that were indeterminate by cytology using standard EUS FNA and ERCP brushing procedures. MP and indeterminate cytology results were compared to patient outcomes derived from surgical pathology or clinical follow-up. The ability of MP to change clinical management recommendations and the impact of those changes on patient outcomes were also examined.

METHODS

Patients

Consecutive patients (>18 years of age) who had endoscopic ultrasound fine needle aspiration (EUS FNA) and/or endoscopic retrograde cholangio-pancreatography (ERCP) evaluation for SPL were included in the study. All patients included had non-diagnostic cytology results (benign, accellular, atypical or suspicious) and MP results at baseline testing. Subjects were excluded if i) a definitive clinical outcome could not be determined from patient follow-up records or patients had less than 12 months of follow-up, ii) patients with a surgical pathology or cytology diagnoses of neuroendocrine tumor or lymphoma or iii) MP results were non-assessable due to insufficient PCR-amplifiable DNA in the specimen. All baseline cytology and MP data and follow-up patient outcomes data were obtained from retrospective chart under IRB approval (Quorum IRB #29760). All clinical data was entered into a de-identified database for analysis.

Cytology

Baseline indeterminate cytology results were grouped into two categories based on language reported. The first category was considered 'non-malignant' and included: i) 'non-diagnostic' cytology due to low cellularity and ii) 'benign' cytology due to no or mild atypia but adequate cells. The second category was considered 'suspicious' due the presence of cells suspicious for malignancy but absence of cells that were frankly malignant. For analysis purposes, patients with 'non-malignant' cytology were considered lower risk and patients with 'suspicious' cytology were considered higher risk.

Mutation Profiling

A clinically validated panel (*PancraGen™*, Interpace Diagnostic, Parsippany, NJ) was used for MP, which included assessment for *KRAS* oncogene point mutations and the presence of allelic imbalance, measured by loss of heterozygosity mutation (LOH), at 10 genomic loci linked to tumor suppressor genes (TSG) associated with pancreaticobiliary cancer: 1p (*CMM1, Lmyc*), 3p (*VHL, OGG1*), 5q (*MCC, APC*), 9p (*CDKN2A, CDKN2B*), 10q (*PTEN, MXI1*), 17p (*TP53*), 17q (*NME1, RNF34*), 18q (*DCC*), 21q (*TFF1, PSEN2*), and 22q (*NF2*) [9]. MP was performed on specimens obtained by standard clinical EUS FNA and ERCP procedures. Specimens analyzed included cytology slides and cell-free supernatant fluid that is normally discarded after cytocentrifugation of cells for cytology cell block preparation. For analysis purposes MP diagnoses were categorized as low risk (Benign or Statistically Indolent) or 'high risk' (Statistically Higher Risk or Aggressive) based on diagnostic language provided in the MP clinical report for each patient that underwent MP testing as part of their standard of care.

Patient Outcomes

Patient outcomes were determined from surgical pathology, follow-up cytology indicating definitive malignancy, or clinical follow-up (oncology records, imaging records, clinic notes, communication with the patients' oncologist and/or primary care physician, or death records). For analysis, patient outcomes were dichotomized as 'malignant' or 'benign'. Malignant outcomes included any of the following: i) surgical pathology results of adenocarcinoma and/or high-grade dysplasia, ii) cytology diagnosis of malignant cells and/or adenocarcinoma at a follow-up procedure, or iii) pancreaticobiliary cancer diagnosis, cancer treatment, or cancer as a cause of death in follow-up records. Benign outcomes included any of the following: i) surgical pathology results of negative for malignancy or dysplasia, low-grade dysplasia, and/or intermediate-grade dysplasia or ii) clinical follow-up without indication of malignant diagnosis for at least 12 months after baseline testing.

Clinical Utility Assessment

All patients from the retrospective chart review were included in a clinical utility assessment by two clinical gastroenterologists who specialize in EUS and ERCP procedures. Clinicians were blinded to patient outcomes through de-identification of clinical files that were prepared for each patient. Initially, the clinical files contained clinical, imaging and cytology reports; MP diagnoses and final outcomes were not included. Each investigator separately reviewed the files of the each patient and answered questions regarding treatment recommendations. Two categorical questions were asked: i) "What management course would you recommend" with options including "No surveillance or treatment for cancer", "Active surveillance", or "Treatment for cancer" and ii) "How confident are you with this recommendation" with options including "Very confident", "Somewhat confident", or "Not at all confident". After a one month re-blinding period, the MP report but not patient outcomes were included in the same clinical files, and the clinicians were again asked to answer the same questions. For analysis, management recommendations were categorized as conservative (i.e. "No surveillance or treatment for cancer" or "Active surveillance") or aggressive (i.e. "Treatment for cancer"). The level of confidence in each recommendation was categorized as confident (i.e. "Somewhat confident" or "Very confident") or not confident (i.e. "Not at all confident"). Management recommendations and the level of certainty in those management recommendations were compared before and after MP results were included in clinical files.

Statistics

The performance characteristics of indeterminate cytology (categorized as non-malignant or suspicious), MP (categorized as low or high risk) and the combination of cytology and MP (categorized as high risk if cytology was suspicious and/or MP was high risk and low risk if both cytology was non-malignant and MP was low risk) were examined by 2×2 contingency table analysis. Statistical differences in sensitivity and negative predictive value were calculated using McNemar's test and a weighted generalized scoring statistic, respectively. Logistic regression analysis was used to identify predictors of benign outcomes.

Bayes theorem was used to calculate the projected absolute risk of malignancy based on MP results at variable baseline probabilities of malignancy using the performance of MP in the study cohort. Projected risk of malignancy was examined in a hypothetical cohort of 1000 patients for each baseline probability. The absolute risk of malignancy imparted by MP results were compared to the baseline probability of malignancy [10]. All calculations assumed conservation of intrinsic parameter performance in distinct test populations. Statistical significance in the relative risk of malignancy based on MP results compared to baseline probability of malignancy was assessed using Pearson's chi-square test.

McNemar's test was used to evaluate significant changes in management choices (i.e. conservative *vs*. aggressive treatment) and confidence level of management recommendations (i.e. not confident *vs*. confident) before and after MP results were reviewed. Logistic regression analysis was used to assess the significant impact of MP results on changes in management choices.

RESULTS

A total of 232 patients (44% men) with indeterminate cytology results (i.e. non-diagnostic, benign, atypical or suspicious) were included in the study cohort (mean age 66.9 years, SE, 0.9). Sixteen patients were excluded due to i) non-definitive clinical outcome or less than 12 months of follow-up (n=9), ii) neuroendocrine tumor or lymphoma (n=2), or iii) non-assessable MP results (n=5).

The mean size of SPLs was 2.2 cm (SD, 1.1 cm). Features of chronic pancreatitis were present upon EUS in 41 (17.7%) patients. In 157 (67%) patients the mass was in the head of the pancreas. Dilated main pancreatic duct in relation to the mass was present in 47 (20%) patients, cystic solid component in 68 (29%) patients, obstructive jaundice in 26 (11%), and abdominal pain with weight loss in 168 (73%). Forty-nine patients (21%) had confirmed pancreaticobiliary malignancy at a median follow-up time of 17.2 months.

At baseline testing, non-malignant cytology results were reported in 207 (89%) patients, including 112 with non-diagnostic (i.e. accellular) cytology and 95 with benign or atypical cells. Cytology was suspicious in only 25 patients at baseline testing. Eventually, malignancy was confirmed in 14% of patients with non-malignant cytology (86% NPV, **Table 1**), occurring in 23% of those with non-diagnostic cytology and 3% of those with benign or atypical cells. Malignancy was confirmed in 80% of patients with suspicious cytology (80% PPV, **Table 1**).

MP was performed on specimens from 218 (94%) patients that had EUS FNA and 14 (6%) patients that had ERCP bile duct brushings. High risk MP results were present in 42/232 patients, including 23/25 patients that had suspicious cytology and 19/207 patients that had non-malignant cytology results. Low risk MP results were present in 190/232 patients. KRAS mutation was detected in 28/49 malignancies and TSG LOH mutation in 19/49. Sixteen malignancies had KRAS mutation but not TSG LOH mutation, and 7 malignancies had TSG LOH mutation but not KRAS.

When cytology was non-malignant, MP detected 45% of malignancies with high specificity. MP detected 46% in the subset of non-diagnostic (i.e. accellular) cytology cases **(Table 1)**. Compared to use of cytology alone, the presence of either suspicious cytology or high risk MP results improved sensitivity (41% *vs*. 67%, p<0.001) and the absence of both improved negative predictive value (86% to 92%, p<0.05). In a logistic regression (LR) model low risk MP results were a strong predictor of benign outcome (29 OR, p<0.0001, **Table 2**).

Prior to second-line DNA analysis, first-line cytology and imaging results triage patients into subgroups with variable cancer probabilities. We performed sensitivity analysis to better understand the predictive value of MP in such scenarios using the sensitivity and specificity of MP observed in our study cohort **(Table 1)**. MP was useful in distinguishing patients at higher risk of malignancy from those at lower risk over a range of cancer probabilities **(Figure 1)**. Compared to the risk of malignancy associated with non-malignant cytology (3-23%), patients reclassified as high risk by MP were at 4-10 fold higher risk of malignancy (30-80%, both p<0.001). Patients reclassified as low risk by MP were at 0.4 fold lower risk of malignancy relative to patients with non-diagnostic cytology (9% *vs*. 23% risk, p=0.012) **(Figure 1)**. Compared to risk associated with suspicious cytology (80%), patients reclassified as high risk by MP were at fold 1.2 fold higher risk (98%, p<0.001) and patients reclassified as low risk by MP were at 0.7 fold lower risk (58%, p=0.001).

As part of the study we evaluated the ability of MP to change clinician management recommendations. We also examined the impact of those recommendations on patient outcomes. Management recommendations made based on cytology and imaging results for the 232 patients in the study cohort were compared to recommendations made based on cytology, imaging and the addition of MP results. After viewing MP results, clinicians changed their initial management recommendation from more aggressive treatment for cancer to more conservative surveillance in 10% of cases (p<0.04). Furthermore, clinicians more frequently reported higher confidence in

Table 1. Performance characteristics of indeterminate cytology and MP in patients with a solid pancreaticobiliary lesion (SPL).

Test*	% Sensitivity (95% CI)	% Specificity (95% CI)	% PPV (95% CI)	% NPV (95% CI)	PLR (95% CI)	NLR (95% CI)
Cytology only (n=232)	41 (27-56)	97 (94-99)	80 (59-93)	86 (81-90)	15 (6-38)	0.61 (0.5-0.8)
Cytology combined with MP (n=232)	67 (53-80)	95 (90-97)	81 (65-91)	92 (81-95)	12 (6-23)	0.35 (0.2-0.5)
MP in cases of non-malignant cytology (n=207)	45 (27-64)	97 (93-99)	68 (47-84)	92 (89-94)	13 (6-32)	0.57 (0.4-0.8)
MP in cases of non-diagnostic cytology (n=112)	46 (27-67)	94 (87-98)	71 (48-86)	85 (77-92)	8 (3-21)	0.57 (0.4-0.8)

Table 2. Logistic regression showing predictors of benign outcome in patients with a solid pancreaticobiliary lesion (SPL) that has indeterminate cytology.

Predictors	Odds Ratio (OR)	95% Confidence Interval	P value
Age	1.024	0.981-1.069	0.28
Absence of obstructive jaundice	1.989	0.57-7.18	0.29
Mass <2 cm in size	1.331	0.48-3.69	0.58
Absence of dilated main pancreatic duct	0.64	0.22-1.92	0.43
Absence of suspicious cytology	2.52	0.49-12.81	0.27
Benign IMP	29.5	7-118	0.0001

Table 3. Bivariate logistic regression showing predictors of recommendations for conservative management of patients with a solid pancreaticobiliary lesion (SPL) that has indeterminate cytology.

Predictors	Odds Ratio (OR)	95% Confidence Interval	P value
Age	0.96	0.91-1.004	0.07
Absence of obstructive jaundice	12.2	2.7-54	0.001
Mass <2 cm in size	5.8	1.5-22	0.01
Absence of dilated main pancreatic duct	2.5	0.78-7.9	0.13
Absence of suspicious cytology	2.1	0.3-15.4	0.45
Benign IMP	131	20-839	0.0001

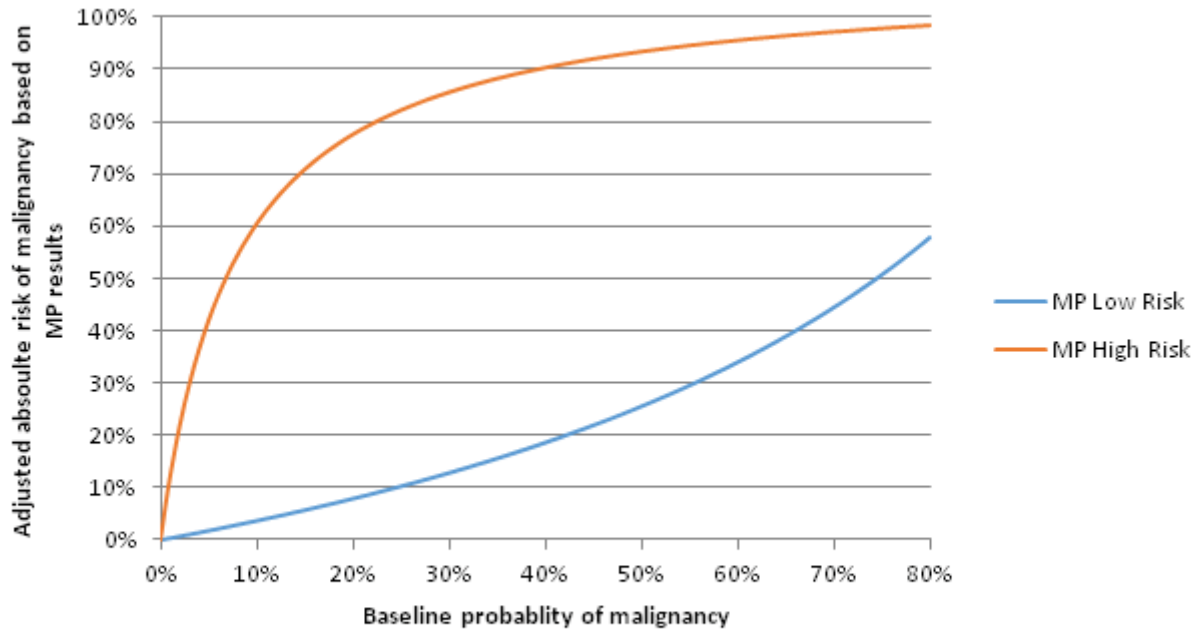

Figure 1. Sensitivity analysis showing the adjusted absolute risk of malignancy given low or high risk MP results over a range of cancer probabilities. Absolute risk of malignancy was calculated using Bayes theorem incorporating the performance characteristics of MP in all solid pancreaticobiliary lesions (SPLs) with indeterminate cytology from the study cohort (n=232). MP mutation profile.

their recommendations after viewing MP results (98%) compared to before (91%) (p<0.017). Mutual agreement in management recommendations among the clinicians also improved after viewing MP results (0.71 kappa) compared to before (0.56 kappa).

Importantly, the average rate of benign disease in patients recommended for more conservative management increased from 84% to 92% after viewing MP results (p<0.05). The average rate of malignant disease in patients recommended for more aggressive management increased from 53% to 71% (p<0.05). In logistic regression analysis, low risk MP results were strongly predictive of recommendations for conservative management (130 OR, p<0.0001, **Table 3**). The absence of obstructive jaundice or small lesion size (<2 cm) were the only other significant predictors of conservative management.

DISCUSSION

For solid pancreatic lesions (SPLs), early and definitive cytological diagnosis is crucial and EUS guided FNA has become the most common method of obtaining a tissue diagnosis. However despite significant advances in needle devices including the ability to take core biopsy samples, and also, availability of ancillary imaging technology such as contrast-enhanced EUS and elastography, indeterminate cytology remains a common problem. Most of the reports of high diagnostic accuracy of EUS FNA are currently from centers of excellence, performed by expert endosonographers and interpreted by expert cytopathologists. However, the proportion of indeterminate cytology samples is likely increased in community based centers, which often do not have the requisite expertise. Consistently, a recent review reported that the false negative diagnostic rate for EUS FNA can be as high as 45% in SPLs [11]. Furthermore, while fine needle core biopsy has the ability to obtain a histological specimen to study tissue architecture, it per se does not improve the diagnostic yield of malignancy compared to FNA [11, 12]. Some of the inherent biological limitations such as paucicellular samples in a desmoplastic stroma typically seen in pancreatic adenocarcinoma, topographic morphological variability in a neoplastic cellular population, and the reactive cellular atypia seen with inflammatory lesions will always limit cytological diagnostic accuracy. Accessibility of the lesion, the endoscopist's expertise in procuring a representative, adequate sample, technician's skill in making an optimal smear, availability of on-site cytological assessment and significant inter-observer disagreement between cytopathologists are other factors that often lead to indeterminate cytology [13].

Newer developments in sequencing technology have led to the ability of high volume sequencing from only small amounts of DNA. Combining MP with cytological assessment may easily obviate the limitation of cytological assessment and help improve its diagnostic accuracy. Considerable published information is available on the diagnostic utility of MP of cells from microdissected slides and cell-free DNA from cytocentrifugation supernatant fluid of EUS FNA and ERCP brushing procedures [7, 14]. However, these studies have been limited by small sample size and have not examined the specific impact of these ancillary diagnostic tests on clinical decision making.

In the current study, the combination of cytology and MP increased overall diagnostic accuracy for malignancy in patients with SPLs with indeterminate cytology results. Compared to use of cytology alone, the presence of suspicious cytology and/or high risk MP results improved sensitivity and the absence of both improved negative predictive value. MP was useful in distinguishing patients at higher risk of malignancy from those at lower risk over a range of cancer probabilities anticipated for indeterminate cytology. However, although high risk MP results were able to help confirm the presence of malignancy in cases in which cytology indicated a high suspicion of malignancy, low risk results could not effectively exclude the possibility of malignancy in such cases.

In our study, two clinicians initially blinded to MP results changed their management recommendation to a more conservative plan in 10% of patients, and these recommendations were made with a higher level of confidence. Importantly, use of MP to aid in decisions increased the rate of benign disease in patients recommended for conservative management and the rate of malignant disease in patients recommended for aggressive management. Such improvements to patient outcomes were obtainable from one initial diagnostic procedure, which can lead to more general cost-effective care.

Interestingly, availability of MP results also led to more concordant management recommendations. Furthermore, low risk MP results decreased the relative uncertainty associated with conservative management. These results are very relevant given that a recent consensus statement by a group of expert pancreatic surgeons has reported that up to 15% of patients undergo unnecessary pancreatic surgery even in centers of excellence [15]. This proportion is likely to be much higher in community practices. Given our results, incorporation of MP testing into clinical decision making is expected to increase a clinician's level of confidence in recommending conservative management rather than aggressive and expensive surgical procedures to compensate for clinical uncertainty.

CONCLUSION

Our study is limited by its retrospective nature. The performance of MP in combination with cytology and its impact on clinical decision making was based on clinical record review of patients who had MP testing as part of their clinical standard of care. However, the performance of MP in combination with cytology was consistent with that reported by others in a prospective study of solid pancreaticobiliary lesions in which the combination of cytology and MP also had superior diagnostic accuracy compared to use of cytology alone [8].

Many studies have shown that pre-malignant pancreatic lesions, such as Pan-INs, MCNs, and IPMNs, can harbor gene mutations, including those examined by MP. However, not all lesions are malignant. By contrast, we show that in the context of SPLs, the mutations examined by MP were highly specific for cancer. MP identified malignancies that were otherwise not detected by cytology. When used in combination, the presence of suspicious cytology and/or high risk MP results improved sensitivity for malignancy and the absence of both improved negative predictive value. Furthermore, MP results significantly impacted clinical decision making. Low risk MP results led to more confident recommendations for conservative surveillance with higher inter-rater agreement between clinicians. Use of MP in combination with indeterminate cytology results may help to more confidently and appropriately avoid

unnecessary aggressive interventions, such as surgery, in patients with SPLs.

Conflict of Interest/Funding

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SAJ, NAT, JLS, CMN, SDF are employees of Interpace Diagnostics Corporation. No other authors have conflicts of interest to declare.

Author Contributions

Study concept and design: SAJ, NAT, JLS, SDF, CMN, AD

Acquisition of Data: All authors

Drafting of the article: AD, SAJ

Critical revision for important intellectual content: All authors

Final approval of the article: All authors

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